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Morphological, physiological, biochemical and molecular characterization of statin-producing *Penicillium* microfungi isolated from little-explored tropical ecosystems

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ABSTRACT

As hypercholesterolemia is a primary risk factor for coronary artery disease and stroke, there is now an increasing demand for cholesterol-lowering drugs. Statins are a group of extremely successful drugs that lower the cholesterol level in the blood. Natural statins are produced by fermentation using different species of microorganisms. The overall aim of the present study was to identify statin-producing microfungi, which were isolated from different types of little-explored mangrove and oil palm plantation soils. Isolated fungal cultures were characterized on the basis of morphological, physiological, biochemical, and molecular features. Morphological variability was detected amongst the fungal isolates in regard to colony morphology, conidiophores structures, and conidia coloration. Based on their physiological properties and enzyme assays, rapid differentiation of statin-producing isolates was achieved. Further molecular characterization allowed reliable identification of the selected *Penicillium* microfungi up to the species level. The identified *Penicillium cintrinum* ESF2M, *Penicillium brefeldianum* ESF21P, and *Penicillium janthinellum* ESF26P strains have a scientific interest as novel wild-type producers of natural statins.

Abbreviations

HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
PDA	Potato-Dextrose Agar
CZ	Czapek-Dox Agar
CYA25	Czapek Yeast Autolysate Agar
MEA	Malt Extract Agar
YES	Yeast Extract Sucrose Agar
CYAS	Czapek Yeast Autolysate with 5% NaCl Agar
DMSO	dimethylsulfoxide
CD	Colony diameter
COC	Colony obverse color
CRC	Colony reverse color
HPLC	High-performance liquid chromatography.

Introduction

Cholesterol is a steroid molecule involved in many biological processes in the human body. For example, it is an important component of human cell biomembranes and a precursor for steroid hormones. It is also the substrate for the biosynthesis of bile acids essential for the absorption of fats and fat-soluble vitamins from the intestine. Although this compound is an important component of lipid metabolism, high blood cholesterol level (hypercholesterolemia) is causally related to cardiovascular diseases which are considered as the leading cause of death worldwide. Elevated plasma cholesterol levels have long been recognized as a major risk factor of atherosclerosis and coronary artery disease. As only one-third of the total body cholesterol is diet-derived and two-thirds are derived from *de novo* synthesis in humans, the biosynthesis of cholesterol is considered a major contributing factor to hypercholesterolemia (Manzoni and Rollini, 2002; Barrios-González and Miranda, 2010).

Although the cholesterol biosynthetic pathway involves more than 25 enzymes, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) is the rate-limiting enzyme for cholesterol formation in the liver and to a lesser extent in other organs. The natural substrate of this enzyme, HMG-CoA, is a water-soluble compound and there are alternative metabolic pathways for its breakdown when HMG-

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CoA reductase is inhibited so that there is no buildup of lipophilic intermediates with a sterol ring (Manzoni and Rollini, 2002). Statins are a group of effective drugs that lower the cholesterol level in the blood by inhibiting HMG-CoA reductase. They are structural analogues of HMG-CoA and thereby inhibit HMG-CoA reductase competitively with an affinity about 1000–10,000 times greater than that of the natural substrate (Pahan, 2006).

Based on their origin, commercially available statins can be divided into the natural statins, the semi-synthetic, and synthetic statins. The natural statins share very similar chemical structure. In contrast to the natural statins, the chemical structures of the synthetic statins are both individually dissimilar and different from the natural forms. However, the HMG-CoA-like moiety, responsible for HMG-CoA reductase inhibition, is common to both natural and synthetic statins (Manzoni and Rollini, 2002).

In addition to cholesterol-lowering properties, several reports have indicated many other biological actions of statins which are summarized in Table 1. From Table 1, it can be seen that in addition to reducing the risk of cardiovascular morbidity and mortality, these valuable drugs have emerged as possible medicines for many other chronic disorders.

Natural statins can be obtained from different genera and species of microorganisms by fermentation, which decreases production costs compared to chemical synthesis. Lovastatin (also called mevinolin or monacolin K) is mainly produced by different strains of *Aspergillus terreus* and mevastatin (also called ML-236B or compactin) by *Penicillium* strains (Manzoni and Rollini, 2002; Chakravarti and Sahai, 2004; Barrios-González and Miranda, 2010; Subhan et al., 2016). In comparison with other statins, pravastatin is a highly selective natural inhibitor of hepatic cholesterol synthesis. It is currently manufactured by microbial hydroxylation of mevastatin. However, this includes two fermentations and involves a number of limitations (Seydametova, 2013; Syed and Ponnusamy, 2018). A one-step fermentation process for the production of pravastatin could be a more attractive approach from both engineering and economic points of view. Therefore, the continued search for microorganisms that are able to synthesize this natural statin directly is very important.

Table 1
Summary of important effects of commercially available statins.

Effects of statins	References
Lipid-lowering effects	Manzoni and Rollini, 2002 Barrios-González and Miranda, 2010 Seydametova, 2013 Syed and Ponnusamy, 2018
Anti-atherosclerotic effects	Barrios-González and Miranda, 2010 Syed and Ponnusamy, 2018
Antithrombotic effects	Barrios-González and Miranda, 2010 Seydametova, 2013
Neuroprotective properties	Barrios-González and Miranda, 2010 Syed and Ponnusamy, 2018
Antihypertensive effects	Manzoni and Rollini, 2002 Syed and Ponnusamy, 2018
Beneficial effects on bone formation and bone mineral density	Manzoni and Rollini, 2002 Barrios-González and Miranda, 2010 Seydametova, 2013
Immunomodulatory properties in patients with multiple sclerosis	Barrios-González and Miranda, 2010 Syed and Ponnusamy, 2018
Immunomodulatory and anti-inflammatory effects in patients with rheumatoid arthritis	Barrios-González and Miranda, 2010 Seydametova, 2013
Favorable effects on glucose metabolism, insulin secretion and insulin sensitivity	Barrios-González and Miranda, 2010 Syed and Ponnusamy, 2018
Antiproliferative properties	Barrios-González and Miranda, 2010 Seydametova, 2013 Syed and Ponnusamy, 2018
Favorable effects in patients with progressive renal disease	Barrios-González and Miranda, 2010
Suppressive effects on cholesterol gallstone formation	Syed and Ponnusamy, 2018

The known hypocholesterolemic activity of some secondary metabolites from different *Penicillium* species has focused our search for potent statin producers among this taxonomic group of microfungi (Shindia, 1997; Manzoni and Rollini, 2002; Chakravarti and Sahai, 2004; Barrios-González and Miranda, 2010). From the literature, it is known that *Penicillium* species are one of the most commonly occurring soil microfungi associated with the rhizosphere of many plants in natural ecosystems (Carlile et al., 2001). Therefore, we hypothesized that different *Penicillium* strains isolated from rhizosphere soils of plants in mangrove forests might have the ability to synthesize natural statins. Moreover, earlier Muniandy et al. (2009) reported that oil palm plantation soils contain significant amounts of lipid compounds. Therefore, *Penicillium* microfungi isolated from these soils might also produce natural antihypercholesterolemic agents. It should be noted here that until now no substantial efforts have been made to assess the biotechnological potential of soil mycobiota of these unique habitats in the Pahang State of Malaysia. For these reasons, as the initial step in the search for *Penicillium* species capable of synthesizing natural statins, the soil microfungi were isolated by our research group from the rhizosphere of plants naturally growing in mangrove forests as well as from soils collected in oil palm plantations of different ages, which are located in the Pahang State. Then, these fungal cultures were screened for natural statin production. As a result of our screening program, it was found that several fungal isolates produced natural statins at different levels. The purpose of the present study was to characterize and identify these potent statin-producing microfungi employing morphological, physiological, biochemical, and molecular methods.

Materials and methods

2.1. Fungal isolates

The fungal culture ESF2M was isolated from clay soil collected from the rhizosphere of *Rhizophora apiculata* in a mangrove forest in Kuantan (3.79°-N 103.31°-E) in the Pahang State of Peninsular Malaysia. The fungal isolates ESF21P and ESF26P were recovered from peat soil samples collected from a 15-year-old oil palm plantation in Gambang (3.72°-N 103.12°-E) in the Pahang State (Malaysia).

2.2. Morphological identification of the fungal isolates

Filamentous fungi, initially isolated by plating collected soil samples on Potato-Dextrose Agar (PDA), were transferred to appropriate diagnostic agar media for identification (Pitt, 1979; Frisvad and Samson, 2004): 1) Czapek-Dox (CZ) Agar; 2) Czapek Yeast Autolysate (CYA25) Agar; 3) Malt Extract Agar (MEA); 4) Yeast Extract Sucrose (YES) Agar. Fungal cultures were incubated at 25 °C for 7 d Colony diameter, as well as colony obverse and reverse colors, were recorded directly from the Petri dishes. Micro-morphological observations were made in accordance with a standard procedure after incubation of the fungal isolates on MEA medium at 25 °C for 7 d Microscopic characteristics such as conidiophore branching patterns were examined using an optical microscope (Primo Star Carl Zeiss, Germany). These techniques allowed the fungal isolates to be identified to the genus level.

2.3. Physiological characterization

The growth of the fungal isolates on CYA agar medium at 25 °C (CYA25) and 30 °C (CYA30) as well as on Czapek Yeast Autolysate with 5% NaCl (CYAS) agar medium was determined as described in the literature (Pitt, 1979; Frisvad and Samson, 2004).

2.4. Enzyme assays

The enzymatic activities of the tested fungal isolates were evaluated using the API ZYM system (BioMerieux SA, France). The experimental procedure applied followed the manufacturer's instructions as slightly modified by Bridge and Hawksworth (1984) and was as follows. Isolates to be tested were grown on CZ agar at 25 °C for 7 d, and a spore suspension of each isolate was prepared in an aqueous solution of Tween-80 (0.2%, v/v) to an opacity equivalent to that of a MacFarland No. 4 standard (approximately 2×10^6 spores/mL). Then, 65 μL of the suspension were added to each of the 20 cupules (1 for control and 19 for substrates) in the API ZYM strip, which was placed in a plastic chamber moistened with 5 mL of water. The chamber was covered with a lid and incubated at 37 °C for 6 h. After the incubation period, 1 drop of each of the ZYM reagents A and B was added to each of the cupules. Reagent ZYM A contains 25 g of Tris-hydroxymethyl-aminomethane, 11 mL of 37% hydrochloric acid, 10 g of sodium lauryl sulfate, and distilled water to make up a volume of 100 mL (pH 7.6 to 7.8). Reagent ZYM B contains 0.12 g of Fast Blue BB (active ingredient), 40 mL of methanol, and 60 mL of dimethylsulfoxide (DMSO). The color intensity developed after 5 min in the dark was graded from 0 to 5 with reference to the API ZYM color chart: 0 corresponds to a negative reaction, 5 to a reaction of maximum intensity and values 1, 2, 3 or 4 are intermediate reactions depending on the level of intensity (3, 4 or 5 being considered as positive reactions). All tested strains were examined twice to control the reproducibility of the results.

For numerical analysis of the obtained results, the enzyme activities observed for each fungal isolate were coded as follows: 0 – negative reaction; 1 – moderate reaction; 2 – strong positive reaction. The coded results for all strains were combined and compared. The data were examined with the SPSS 16.0 cluster program, using the unweighted centroid linkage method. Euclidean (amalgamation) distance was used to combine clusters and draw a dendrogram. The dendrogram contained clusters, grouping the strains with similar enzymatic profiles.

2.5. Molecular identification

The pure isolates were cultured in 100-mL conical flasks containing 20 mL of CYA liquid medium. Mycelia from 2-ᴅ-old cultures were harvested by filtration through Whatman filter paper No. 1 and used for DNA isolation. Genomic DNA was obtained using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation, Canada), according to the manufacturer's instructions. The ITS1–5.8S-ITS2 region of rDNA from the isolates was amplified using universal fungal primers ITS1 (forward) and ITS4 (reverse) (White et al., 1990). All amplification reactions were carried out in volumes of 25 μL containing 4 μL (10 ng) of template DNA, 1.5 μL of each primer (20 μM), 2.5 μL of 10 × PCR buffer, 1 μL of $MgCl_2$ (50 mM), 0.25 μL of dNTPs (100 mM) and 0.25 μL of Taq DNA polymerase (5 U/μL) (Patino et al., 2007). The amplification program included one cycle of 4 min 30 s at 95 °C (predenaturing), 40 cycles of 30 s at 95 °C (denaturation), 30 s at 50 °C (annealing), 60 s at 72 °C (extension) and finally one cycle of 3 min at 72 °C. For each PCR reaction, 5 μL of PCR product was examined by electrophoresis at 90 V for 1 h 50 min in a 1% (w/v) agarose gel stained with GoodView nucleic acid stain (Beijing SBS Genetech Co. Ltd., China) in 1 × TAE buffer in a Sub-Cell GT agarose gel electrophoresis system (Bio-Rad Laboratories, USA). The molecular masses of the amplified DNA were estimated by comparison with a ready-to-use VC 100-bp Plus DNA ladder (Vivantis Technologies, USA) as a molecular size marker. The agarose gels were visualized under UV light using an Alpha Ease FC Imaging system (Alpha Innotech, Germany).

The purified PCR products were sent to the service provider (First Base Laboratories Sdn Bhd, Malaysia) for sequencing. The sequences obtained were pairwise aligned using the SDSC Biology Workbench 3.2 software with default settings (Subramaniam, 1998). For the identification of fungal isolates, the percentage of coverage and sequence identity were compared with available sequences in GenBank using the Basic Local Alignment Tool, BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to search for the closest matched sequences.

The sequence data from fungal cultures, identified in this study based on the ITS1–5.8S-ITS2 region of rDNA, were deposited in the GenBank database under the following NCBI accession numbers: *Penicillium citrinum* ESF2M (JX442545), *Penicillium brefeldianum* ESF21P (JX462555), and *Penicillium janthinellum* ESF26P (JX863411).

2.6. Phylogenetic analysis

Phylogenetic analysis was performed from aligned ITS1–5.8S-ITS2 sequences. The sequences were aligned using ClustalW with all multiple alignment parameters used at default settings. The sequence alignment included the fungal strains selected in this study and representatives of other *Penicillium* species available from GenBank. *Talaromyces bacillisporus* CBS 296.48 (JN899329) was the outgroup species. Cladistic analysis using the neighbor-joining method was performed with the MEGA 4.0 computer program (Tamura et al., 2007). Phylogenetic distances were calculated using the Jukes-Cantor model. Confidence values for individual branches were determined by 1000 bootstrap replications.

Results

3.1. Phenotypic features of the statin-producing fungal isolates

Initially, 20 fungal isolates were isolated by our research group from soil samples collected from the rhizospheres of plants naturally growing in mangrove forests located in Balok, Cherating, and Kuantan of the Pahang State of Peninsular Malaysia: *Avicennia alba, Acrostchum aureum, Excocaria agallocha, Rhizophora apiculata*, and *Bruguiera gymnorrhiza*. In addition, a total of 34 fungal isolates were recovered from the examined sandy, peat, and deep peat soil samples collected in oil palm plantations of different ages (10, 15 and 20 years after planting) in the Pahang State. All recovered fungal isolates were screened for natural statins production after submerged fermentation. Screening of the soil microfungi isolated from these little-explored tropical habitats showed that several fungal cultures produced natural statins at different levels. In particular, the isolate ESF2M recovered from the rhizosphere soil of *Rhizophora apiculata* in a mangrove forest in Kuantan was capable of producing both pravastatin (8.17 mg/L) and lovastatin (20.39 mg/L). The ESF21P and ESF26P strains isolated from peat soil samples collected from a 15-year-old oil palm plantation in Gambang accumulated pravastatin directly at concentrations of 196.83 mg/L and 13.89 mg/L, respectively.

Table 2 shows the cultural properties of the selected isolates, and Table 3 describes their morphological properties. The cultural and microscopic features of the isolates obtained indicated that they were characterized by macromorphology and microscopic elements typical of the genus *Penicillium*.

3.2. Physiological separation of the fungal isolates

The present investigation also aimed to determine the physiological characteristics of the selected statin-producing *Penicillium* microfungi. Table 4 summarizes the physiological data of the tested fungal isolates.

3.3. Enzyme activities of the fungal isolates

The enzymes detected in the present study by the API ZYM system

Table 2
Cultural properties of the statin-producing fungal isolates.

Cultural characteristics	ESF2M	ESF21P	ESF26P
CZ			
CD, mm	11–12	11–13	17–18
COC	green	white to cream	dull grayish-green
CRC	yellow	bright yellow	yellowish-brown
CYA25			
CD, mm	19–20	24–27	31–32
COC	green	white to cream	grayish-green
CRC	yellow	yellow-orange	yellowish-brown
MEA			
CD, mm	14–15	50–52	40–42
COC	green	glaucous	greenish-gray
CRC	yellow	yellow-orange	yellowish-brown
YES			
CD, mm	22–25	35–37	26–27
COC	green	glaucous	glaucous
CRC	yellow	yellow-orange	yellowish-orange

aAll measurements are presented as extremes.
bAbbreviations: CD - colony diameter; COC - colony obverse color; CRC - colony reverse color.

Table 3
Morphological properties of the statin-producing fungal isolates.

Morphological characteristics	ESF2M	ESF21P	ESF26P
Conidiophore pattern	monoverticillate	monoverticillate	monoverticillate
Stipe length (μm)	21.1	31.1	31.0
Phialide shape	ampulliform	flask-shaped	ampulliform
Phialide length (μm)	11.2	11.7	8.6
Conidia size (μm)	2.0 × 1.8	2.3 × 2.3	2.0 × 1.2

Table 4
Physiological characteristics of the examined *Penicillium* spp. microfungi as an aid for fungal isolates differentiation.

Character	ESF2M	ESF21P	ESF26P
CD (CYA25), mm	19	27	31
CD (CYA30), mm	16	31	38
CD (CYAS), mm	15	6	16
Ratio CYAS/CYA25	0.79	0.22	0.52
Ratio CYA30/CYA25	0.84	1.15	1.23

aThe results presented are means from three replicates.
bAbbreviations: CD - colony diameter; CYA25 - growth on Czapek Yeast Autolysate Agar at 25 °C for 7 d; CYA30 - growth on Czapek Yeast Autolysate Agar at 30 °C for 7 d; CYAS - growth on Czapek Yeast Autolysate with 5% NaCl Agar at 25 °C for 7 d

are listed in Table 5. The results of API ZYM tests obtained in the present study were used for numerical analysis. Fig. 1 shows the dendrogram obtained by clustering the isolates with the centroid clustering method, taking into account the results of enzymatic activities for all isolates presented in Table 5. In the dendrogram a clear grouping of the strains with similar enzymatic profiles is apparent.

3.4. Molecular identification of the fungal isolates

Because the DNA region that encodes the 5.8S gene of the ribosomal RNA and the two intergenic spacers ITS1 and ITS2 is well characterized in fungi, as the first step in the present study genomic DNA from the selected statins producers was extracted. The universal fungal primers ITS1 and ITS4 were then used to amplify from the 18S ribosomal RNA gene, through the internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and into the 28S ribosomal RNA gene. All of the fungal strains that were used in this study amplified successfully producing a single PCR product of the desired length, approximately 550–600 base pairs.

Each purified amplicon was double-stranded sequenced. The pairwise aligned sequence data from each fungal isolate were then compared with available sequences in the GenBank databases using the BLASTN program. The rDNA sequence data obtained were compared with DNA sequences deposited in GenBank from known *Penicillium* species. The similarity values obtained ranged from 98 to 99%. From Table 6, the ITS1–5.8S-ITS2 sequence of amplicon from the isolate ESF2M showed the greatest homology (99%) to that of *Penicillium citrinum* KACC43900 (EU821333.1). The nucleotide sequence of the isolate ESF21P showed 99% identity with the sequence of *Penicillium brefeldianum* 3352 (FJ527878.1). The sequence of the ESF26P amplicon showed 98% identity to *Penicillium janthinellum* CBS:340.48 (GU981585.1).

3.5. Phylogenetic studies

Sequencing of the ITS1–5.8S-ITS2 region of rDNA is considered a reliable strategy for fungal phylogenetic relationship analysis. For this reason, the phylogenetic tree was constructed, and the evolutionary relationships of the statin-producing fungal strains with other relevant *Penicillium* species are presented in Fig. 2.

Discussion

Filamentous fungi are one of the most common microorganisms in both uncultivated and cultivated soils and have been isolated from numerous soils worldwide (Carlile et al., 2001). Some fungal genera have representative species in most soils. For example, the genera *Penicillium, Aspergillus, Fusarium*, and *Trichoderma* occur very frequently.

Previously, several fungal genera have been reported to be able to produce natural statins by fermentation. Lovastatin and mevastatin are produced by fermentation, using microorganisms of different species belonging mostly to the genera *Aspergillus* and *Penicillium*, respectively (Shindia, 1997; Chakravarti and Sahai, 2004; Subhan et al., 2016). Pravastatin is currently produced by microbial hydroxylation of its precursor, mevastatin, which is normally synthesized by *Penicillium* species. This bioconversion can be carried out by a number of microorganisms, belonging to the genera *Streptomyces, Nocardia, Actinomadura, Bacillus* and others (Seydametova, 2013; Syed and Ponnusamy, 2018).

Although filamentous fungi are commonly distributed in soils, our knowledge of fungal diversity in tropical ecosystems such as mangrove forests and oil palm plantations is limited (Carlile et al., 2001; Kirk et al., 2004). The soils from these ecosystems can be considered as one of the excellent sources of unique microorganisms. The microfungi isolated from these little-explored tropical habitats might include many unstudied fungal species that could provide biotechnologically valuable compounds. It should be noted that in previous research papers on the microbiota of natural mangrove ecosystems, most attention has been given to the examination of bacterial community composition (Liang et al., 2007; Bharathkumar et al., 2008). In the few available studies on the mycobiota of mangrove areas, some researchers have focused on the investigation of fungi on wood, whilst others have addressed their attention to the diversity of fungal endophytes isolated from mangrove plants (Alias et al., 1995; Xing and Guo, 2011). Because until now no substantial efforts have been made to assess the biotechnological potential of the rhizosphere soil mycobiota in mangrove forests of Pahang State (Malaysia), it was interesting for our research group to investigate the statin-producing capability of the *Penicillium* microfungi isolated from these unique habitats. Moreover, as soils in oil palm plantations contain significant amounts of organic matter including lipid compounds (Muniandy et al., 2009), there was a high probability that filamentous fungi isolated from these cultivated soils might produce increased amounts of natural lipid-lowering agents.

For these reasons, initially, a total of 54 fungal strains were isolated by our research group from rhizosphere soil samples collected in three mangrove forests and in oil palm plantations of different ages, which are

Table 5
Enzymes detected by the API ZYM test.

No.	Enzyme	ESF2M	ESF21P	ESF26P	*Penicillium citrinum* ESF19M	*Penicillium janthinellum* ESF20P
1	Control	n	n	n	n	n
2	Alkaline phosphatase	m	p	p	m	p
3	Esterase (C 4)	p	p	p	p	p
4	Esterase lipase (C 8)	p	p	p	p	p
5	Lipase (C 14)	m	m	m	m	m
6	Leucine arylamidase	p	m	p	p	p
7	Valine arylamidase	n	n	n	n	n
8	Cystine arylamidase	n	m	n	n	n
9	Trypsin	n	n	n	n	n
10	α-chymotrypsin	n	n	n	n	n
11	Acid phosphatase	p	p	p	p	p
12	Naphthol-AS-BI-phosphohydrolase	p	p	p	p	p
13	α-D-galactosidase	p	p	p	p	p
14	β-D-galactosidase	m	p	m	p	m
15	β-D-glucuronidase	m	n	n	m	n
16	α-D-glucosidase	p	p	p	p	p
17	β-D-glucosidase	p	p	p	p	p
18	N-acetyl-β-D-glucosaminidase	p	p	p	p	p
19	α-D-mannosidase	p	p	m	p	m
20	α-L-fucosidase	n	n	m	m	n

aAbbreviations: n - negative reaction (0); m - moderate reaction (scale 1 or 2); p - positive reaction (scale 3–5).
b*Penicillium citrinum* ESF19M (Seydametova et al., 2015) and *Penicillium janthinellum* ESF20P (Seydametova, 2015) were used as reference cultures.

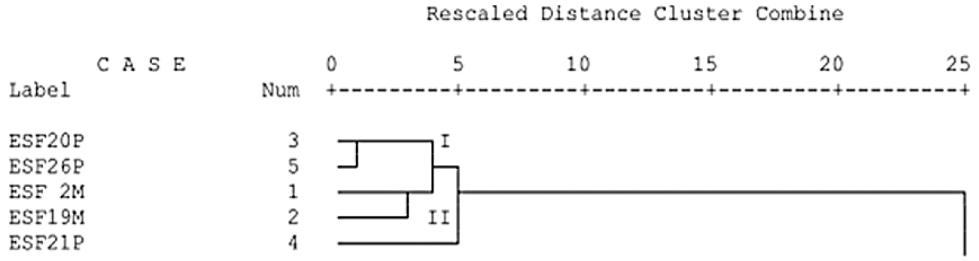

Fig. 1. Dendrogram based on the Euclidean distance and centroid clustering method showing the relationship between clusters of *Penicillium* isolates.

Table 6
Identified fungal isolates with relationship to the species listed in the National Center for Biotechnology Information (NCBI).

Fungal isolate	Closely related sequence	Query coverage (%)	E value	Maximum similarity (%)
ESF2M	*Penicillium citrinum* KACC43900 (EU821333.1)	96%	0.0	99%
ESF21P	*Penicillium brefeldianum* 3352 (FJ527878.1)	96%	0.0	99%
ESF26P	*Penicillium janthinellum* P1 (JQ727998.1)	95%	0.0	98%

located in the Pahang State (Malaysia). Screening of these microfungi showed that the isolates ESF2M, ESF21P, and ESF26P produced pravastatin at different levels. The highest concentration of pravastatin (196.83 mg/L) obtained from the wild-type fungal culture ESF21P was comparable with known active producers of this statin reported in the literature. Manzoni et al. (1999) reported that the *Monascus paxii* AM12M strain, isolated as a spontaneous mutant macro-morphologically different from the original *M. paxii* AM12 strain, produced pravastatin at a level of 45 mg/L after 14 d of fermentation. Several *A. terreus* strains were also capable of producing pravastatin: 121 mg/L for the BST strain, 204 and 34 mg/L for A1R and A2R strains, respectively. In comparison with previous studies, the fungal isolate ESF21P can already be considered as a novel potent producer of pravastatin.

It is noteworthy that when all fungal isolates in our study were examined for mevastatin production using high-performance liquid chromatography (HPLC), no traces of this pravastatin precursor were found in the ethyl acetate extracts of any of the cultures. Being a hydroxylated derivative of mevastatin, pravastatin can commonly be produced by growing specific microorganisms in culture media containing mevastatin in addition to nutrients (Chakravarti and Sahai, 2004). The results obtained here suggest that pravastatin-producing strains selected in our study immediately transformed any mevastatin produced into pravastatin.

It is interesting that the isolate ESF2M was capable of producing both pravastatin and lovastatin. Previously, similar observations have been reported for *M. paxii* AM12M and for *A. terreus* BST strains by Manzoni et al. (1999). This dual production ability might be related to the similar metabolic biosynthetic pathways of the precursors of pravastatin and lovastatin in these fungal isolates.

The overall aim of the present study was to characterize and identify the isolates ESF2M, ESF21P and ESF26P selected during our screening work as potent statin producers. For centuries, fungal identification has been based on phenotypic, physiological, and biochemical characteristics of specimens. For the most part, these systems work well. They provide fungal identification inexpensively, are not labor-intensive, and require little equipment beyond a microscope and chemical reagents. Nowadays, molecular methods are also widely used to distinguish between closely related fungal species.

Phenotypic characters, such as cultural properties, morphology, and growth on selected media have traditionally formed the basis for fungal identification. Therefore, the cultural properties of the selected statin-producing fungal isolates were studied on several diagnostic agar media (CZ, CYA25, MEA, YES) following the procedures described in the literature (Pitt, 1979; Frisvad and Samson, 2004). The morphological features of these microfungi were also examined and included the

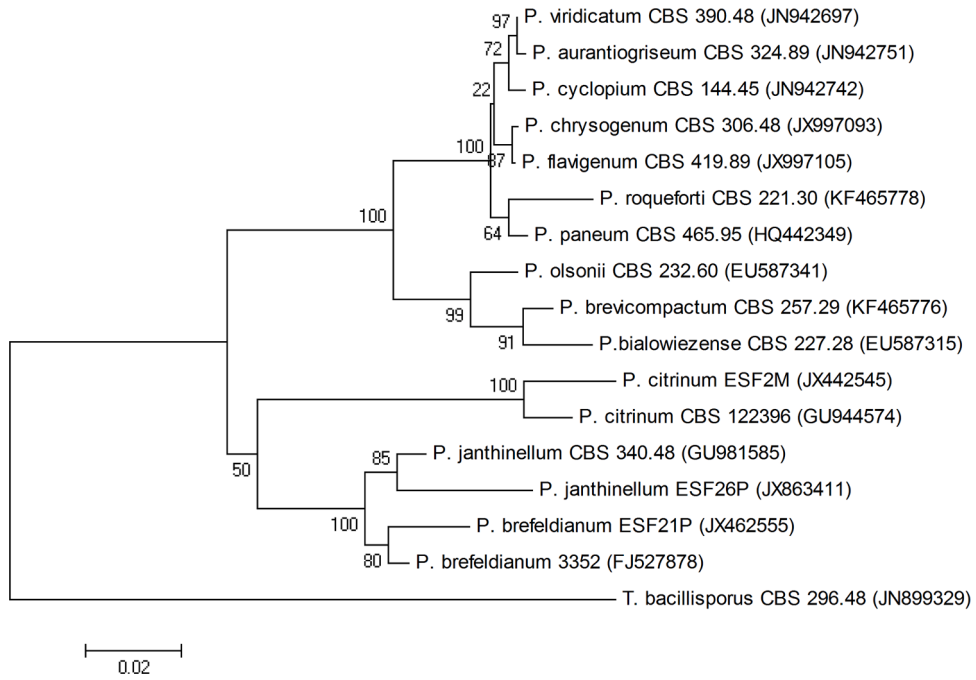

Fig. 2. Phylogenetic tree using the neighbor-joining method of representatives of *Penicillium* species inferred from analysis of the ITS1–5.8S-ITS2 region of rDNA. NCBI accession numbers of the *Penicillium* species are presented in the brackets.

pattern of conidiophores, as well as the shape and size of phialides and conidia. From Tables 2 and 3, the macro- and micro-morphological characteristics of the isolate ESF2M were broadly in agreement with the literature description of *P. citrinum* species (Bridge et al., 1989; Seydametova et al., 2015). The isolate ESF21P most closely resembled *P. brefeldianum* (Stolk and Samson, 1983; McNeill et al., 2012). In the case of ESF26P, the cultural and morphological properties of this isolate were clearly similar to the literature description of *P. janthinellum* (Matsukuma et al., 1994; Seydametova, 2015).

Physiological criteria have not been used extensively in *Penicillium* microfungi identification to date because they in general have sufficiently varied phenotypic characters to allow fungal identification to the genus and even to the species level. However, although the criteria of growth on some diagnostic media introduced by Pitt (1979) are of value in the genus *Penicillium* as a whole, they are of limited value in the subgenus level because some isolates show very similar growth on these media. As indicated by Onions et al. (1984), some *Penicillium* species are especially difficult to identify using classical cultural and morphological criteria alone because of the absence of distinct characteristics. Moreover, the colony texture and color used in *Penicillium* microfungi identification are often subjective criteria. Therefore, physiological criteria are also used to assist in the differentiation of the *Penicillium* microfungi. In the present study, to separate selected statin-producing fungal isolates (Table 4), the colony diameters achieved at different temperatures and water activities were used as described in the literature (Pitt, 1979; Frisvad and Samson, 2004).

In addition to physiological criteria, further examination of the enzymatic activities of the tested fungal strains was evaluated using the API ZYM system in order to differentiate between selected statin-producing isolates. It is noteworthy that previously identified *P. citrinum* ESF19M (Seydametova et al., 2015) and *P. janthinellum* ESF20P (Seydametova, 2015) were also included in these biochemical tests as reference cultures. From Table 5, the strongest activities in all isolates were shown by eight enzymes: esterase (C 4), esterase lipase (C 8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-D-galactosidase, α-D-glucosidase, β-D-glucosidase, and N-acetyl-β-D-glucosaminidase. The following enzymes could not be detected in any of the tested isolates: valine arylamidase, trypsin, and α-chymotrypsin. In relation to enzymatic activities, the data obtained in this study for the fungal cultures ESF2M and ESF26P are generally consistent with those reported in the literature for known *P. citrinum* and *P. janthinellum* species, respectively (Bridge and Hawksworth, 1984). The similarities of the API ZYM enzymatic profiles of the isolates ESF2M and ESF26P with previously identified *P. citrinum* ESF19M (Seydametova et al., 2015) and *P. janthinellum* ESF20P (Seydametova, 2015), respectively, confirmed their close relationship. In spite of this, from API ZYM results presented in Table 5 for the isolate ESF2M, it can be seen that there were little differences in the activities of β-D-galactosidase and α-L-fucosidase in comparison with those of previously identified *P. citrinum* ESF19M. The enzymatic profile obtained for the isolate ESF26P again showed a small difference in the α-L-fucosidase activity in comparison with that of known *P. janthinellum* ESF20P. This small enzymatic variability between the examined isolates and the reference cultures could be explained by the different sources from which they were recovered. The differences found between closely related isolates allowed them to be identified as different strains of the same species. Although Bridges and Hawksworth (1984) have applied the API ZYM system for the characterization of 29 isolates of the genus *Penicillium*, however, until now there have been no literature reports on API ZYM enzymatic profiles of other known species similar to enzymatic activities obtained in the current study for the isolate ESF21P.

The current study has demonstrated that the API ZYM enzyme testing system is a very useful tool to study differences among morphologically similar *Penicillium* strains. Based on the numerical analysis of the obtained results (Table 5), two major clusters (I and II) can be distinguished that show different activity patterns for some enzymes (Fig. 1). Cluster I is subdivided into two subgroups, each of them formed by isolates with similar values for all parameters analyzed. The first subgroup of this cluster contains the previously identified *P. janthinellum* ESF20P species (Seydametova, 2015) and the isolate ESF26P, while the second subgroup consists of known *P. citrinum* ESF19M species (Seydametova et al., 2015) and the isolate ESF2M. Cluster II includes the isolate ESF21P alone, confirming its considerable difference from other tested cultures. Thus, in addition to physiological features, biochemical characteristics provide limited but useful information in the differentiation of closely related *Penicillium* species.

In addition to phenotypic, physiological, and biochemical tools, analysis of the ribosomal region of DNA *via* PCR has been widely employed for characterizing different fungal species, since the ITS1–5.8S-ITS2 region of the rDNA is highly conserved intraspecifically but variable between different species (Carlile et al., 2001). Therefore, all fungal strains tested in the present study were identified to species level using molecular analysis of this region. The sequencing results obtained and their detailed analysis confirmed that the isolate ESF2M belongs to *P. citrinum* species (Table 6). The ESF26P strain was most closely related to *P. janthinellum* species. The isolate ESF21P, which concluded from the phenotypic, physiological, and biochemical studies described above as being more distantly related to the other isolates, showed the highest homology with *P. brefeldianum* species. The high similarity of the sequences from the isolate ESF2M and previously identified *P. citrinum* ESF19M (Seydametova et al., 2015) confirmed their close relationship. In spite of this, minor differences also were found between the sequences which allowed them to be identified as different strains of the same species. The same conclusion can be drawn in terms of the isolate ESF26P which is closely related to known *P. janthinellum* ESF20P (Seydametova, 2015). These observations were again in agreement with the clustering analyses described above based on the results of the API ZYM test.

Conclusion

In the present study, several potent statin-producing microfungi obtained from natural rhizosphere soil of *Rhizophora apiculata* in the mangrove forest and from oil palm plantation soils were characterized. A range of standard macro- and micro-morphological characters of the isolated microfungi were documented. This approach confirmed that all selected fungal isolates were members of the genus *Penicillium*. The results presented here showed that physiological and biochemical tests can also be used as an aid to the rapid differentiation of these *Penicillium* isolates. In addition, the method based on the PCR amplification of ITS1–5.8S-ITS2 region of rDNA followed by sequencing of the amplified fragments proved to be a useful tool for more objective identification of the selected fungal isolates. On the basis of phenotypic and physiological data, as well as the results of the enzyme assays and molecular identification obtained in this investigation, the statin-producing isolates ESF2M, ESF21P, and ESF26P were reasonably proposed to belong to *P. citrinum, P. brefeldianum*, and *P. janthinellum* species, respectively.

Ethical approval

Not required.

CRediT authorship contribution statement

Emine Seydametova: Conceptualization, Methodology, Investigation, Writing – original draft. **Norazwina Zainol:** Funding acquisition, Project administration, Supervision, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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